

Direct evidence of cytoplasmic delivery of PKC- α , - ϵ and - ζ pseudosubstrate lipopeptides: study of their implication in the induction of apoptosis

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Abstract Protein kinases C (PKC) are serine/threonine kinase enzymes involved in the mechanism of cell survival. Their pseudosubstrate sequences are autoinhibitory domains, which maintain the enzyme in an inactive state in the absence of allosteric activators, thus representing an attractive tool for the modulation of different PKC isoforms. Here, we report the use of palmitoylated modified PKC- α , - ϵ , and - ζ pseudosubstrate peptides, and determine their intracellular distribution together with their respective PKC isoenzymes. Finally, we propose that the differential distribution of the peptides is correlated with a selective induction of apoptosis and therefore argues for different involvement of PKC isoforms in the anti-apoptotic program.

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Key words: PKC pseudosubstrate lipopeptide; Intracellular delivery; PKC isoform; Apoptosis

1. Introduction

Protein kinases C (PKCs) are a family of enzymes with serine/threonine kinase activities. They were originally identified as cytoplasmic, calcium-activated, phospholipid-dependent enzymes [1]. Members of this enzyme family have been classified into three groups with respect to their allosteric activators (reviewed in [2]). The conventional PKCs (cPKCs) α , β I, β II, and γ require negatively charged phospholipids, diacylglycerol or phorbol esters and calcium for optimal activation; the novel PKCs (nPKCs) δ , ϵ , θ , η , and μ require negatively charged phospholipids, diacylglycerol or phorbol esters, but not calcium; and the atypical PKCs (aPKCs) λ /I and ζ require only negatively charged phospholipids but not calcium, diacylglycerol or phorbol esters.

Different PKC isoenzymes exhibit a distinct tissue distribution, are activated or inactivated in specific cell compartments by various lipid metabolites, interact with different proteins [3–7] and phosphorylate different substrates which ultimately induce different biological responses [8,9]. PKC is known to play an important role in processes relevant to neoplastic transformation, carcinogenesis and tumor cell invasion. More-

over, among other protein kinases and phosphatases, a key role is indicated for the PKC family in apoptosis.

Apoptosis, or programmed cell death, is a cellular event which underlies a wide variety of normal physiological phenomena, including morphogenesis and limb development [10], clonal selection of lymphocytes [11], and safe removal of inflammatory tissue cells [12]. Knowledge about the role of each PKC isoenzyme in cell survival mechanisms is both limited and controversial [13,14].

In common with other serine/threonine protein kinases, PKC isoenzymes contain an autoinhibitory domain at the N-terminus, in which a short substrate-like peptide, called the pseudosubstrate, binds to the protein-substrate binding region, thus maintaining the enzyme in an inactive state in the absence of allosteric activators [15,16]. These pseudosubstrate sequences appear to be an attractive tool allowing for selective inhibition of different PKC isoenzymes. Recent approaches [17,18], using the myristoylated pseudosubstrate sequence of PKC- α/β , described the modulation of the isoenzyme in a cellular model. In two previous reports we have also described the use of lipid modified PKC- α/β pseudosubstrates to modulate, *in vitro*, the phosphorylation of the purified enzyme [19], and the biological activities related to PKC activation or inhibition [19,20]. In these studies [19,20], biological activities of PKC- α/β pseudosubstrates on intact cells were restricted to lipid modified pseudosubstrate sequences, suggesting an intracellular delivery of the lipopeptide. In the latter report [20], we demonstrated that an N-terminal N^ε-free palmitoyl-lysine-PKC- α/β pseudosubstrate was optimal for providing cytoplasmic delivery of the biologically active lipopeptide. The functionality of the PKC- α/β pseudosubstrate lipopeptide was assessed through the induction of apoptosis in treated cells implying that PKC α/β is involved in cell survival.

In the present report, we used N-terminal palmitoylated PKC pseudosubstrates, designed from the pseudosubstrate sequences of PKC- α , - ϵ , and - ζ , and investigated their cytoplasmic delivery and their ability to selectively modulate their corresponding PKC isoenzymes, by evaluating these PKC pseudosubstrate lipopeptides on purified PKC- α , - ϵ , and - ζ in an *in vitro* enzymatic assay. We also studied the intracellular distribution of the three isoenzymes and their PKC pseudosubstrate lipopeptides in intact cells. Finally, we analyzed the biological relevance of the cytoplasmic delivery of the pseudosubstrate lipopeptides by quantifying their implication in the induction of apoptosis in two different human cell lines.

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2. Materials and methods

2.1. Peptide and lipopeptide synthesis and characterization

We synthesized the lipopeptides K(Pam)DVANRFARKGALRQ, K(Pam)ERMNRPKRQGAVRRRV and K(Pam)SIYRRGARRWRKL, derived respectively from the PKC- α , - ϵ and - ζ pseudosubstrate sequences, modified in the N-terminal position by the addition of a N^ε-palmitoyl-lysine residue. A control 'scrambled' lipopeptide, K(Pam)ASGRLKWRYYRRIR, was designed from the PKC- ζ pseudosubstrate sequence. All lipopeptides were synthesized by the solid-phase method [21] in an automated peptide synthesizer (Applied Biosystems, Model 430A, Foster City, CA, USA) using a standard *t*-butyloxycarbonyl Boc-benzyl strategy as previously described [19]. Boc-protected amino acids were purchased from Propeptide (Vert-Le-Petit, France). Boc-L-lysine(Fmoc) was purchased from Novabiochem (EMA, Meudon, France). Labelled lipopeptide analogs were obtained by addition of a biotin (Sigma, St. Louis, MO, USA), introduced at the N-terminal position before final deprotection with hydrogen fluoride and cleavage from the resin. All compounds were purified by RP-HPLC (Vydac C4 column), checked for homogeneity by RP-HPLC and capillary electrophoresis and were more than 98.5% pure in both systems. They were checked for identity by mass spectrometry (Bio Ion AB, Uppsala, Sweden). PKC- α pseudosubstrate lipopeptide [MH⁺] calc. 1967.4, obs. 1968.3; PKC- ϵ pseudosubstrate lipopeptide [MH⁺] calc. 2417.6, obs. 2417.6; PKC- ζ pseudosubstrate lipopeptide [MH⁺] calc. 2084.6, obs. 2085.2; scrambled lipopeptide [MH⁺] calc. 2084.6, obs. 2083.9.

2.2. In vitro PKC assay

Partially purified recombinant PKC [22] was incubated in a 96 well polystyrene plate in a total volume of 120 μ l in the absence or presence of phosphatidylserine (50 μ M) plus PMA (1 μ M, for PKC- α and - ϵ) diluted in buffer containing 25 mM Tris-HCl (pH 7.0), 3 mM MgCl₂, 0.1 mM ATP, 0.5 mM EDTA, 1 mM EGTA, 2 mM CaCl and 5 mM 2-mercaptoethanol; and in the presence or absence of the specified peptides. After a 30 min pre-incubation period at 30°C, the reaction was started by spotting a 100 μ l of the reaction mixture onto a 96 well substrate-coated plate (Panvera, Madison, WI, USA). The reaction was carried out for 10 min at 30°C.

The amount of phosphorylated substrate was determined by ELISA using a biotinylated monoclonal antibody which recognizes the phosphorylated form of the peptide, according to the manufacturer's protocol (Panvera).

2.3. Cell culture

Jurkat and HL60 human cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Gibco), 2 mM glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin and 100 μ g/ml streptomycin under 5% CO₂ at 37°C.

2.4. Immunofluorescent labelling

The subcellular localization of PKC isoenzymes and the intracellular delivery of PKC pseudosubstrate lipopeptides was assessed using indirect immunofluorescence staining and confocal microscopy. Mouse monoclonal antibodies directed against PKC- α , - ϵ or - ζ were purchased from Transduction Laboratories Inc. (Lexington, KY, USA). Monoclonal goat anti-mouse IgG coupled to Alexa 568, streptavidin conjugated to Alexa 488 and TO-PRO-3 iodide were purchased from Molecular Probes (Leiden, The Netherlands).

Cells (1×10^6) were incubated for 10 min at 37°C with 5 μ M of different biotinylated PKC pseudosubstrate lipopeptides. Cells were washed twice with PBS and then fixed with PBS containing 2.5% paraformaldehyde, 0.01% glutaraldehyde for 30 min at room temperature. Fixed cells were placed on glass coverslips, permeabilized with 0.05% saponin in PBS for 10 min and blocked for 10 min at room temperature in permeabilizing buffer (PBS containing 2% BSA and 0.05% saponin). Then, cells were incubated for 1 h at 37°C with primary antibodies (mouse anti-PKC- α , - ϵ or - ζ) used at 1:50 dilution (5 μ g/ml) in permeabilizing buffer. After five washes with permeabilizing buffer, cells were incubated with both the secondary antibody, a monoclonal goat anti-mouse IgG coupled to Alexa 568 used at a 1:100 dilution (2 μ g/ml) and streptavidin-Alexa 488 conjugate used at 1:100 dilution in permeabilizing buffer. Cells were washed twice with permeabilizing buffer and twice with PBS-2% BSA and then,

nuclear DNA was stained with TO-PRO-3 iodide (0.1 μ M) for 45 min at 37°C.

Slides were mounted using Vectashield (Vector Laboratories, Compiègne, France).

2.5. Confocal microscopy

Fluorescence-stained slides were examined under a Leica TCS NT laser scanning confocal microscope (Leica, Heidelberg, Germany) comprising a krypton/argon laser (488, 568, and 647 nm excitations are possible). Simultaneous three channel recording was performed. Frame scanning was performed at $\times 1000$ magnification and a single optical section was collected per field.

2.6. Detection of apoptosis in Jurkat and HL60 cell lines by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling)

Jurkat or HL60 cell lines (2×10^6 cells) were resuspended in FCS-free medium. Cells were incubated for 6 h with different concentrations of either PKC- α , - ϵ , or - ζ pseudosubstrate lipopeptides. After three washes with PBS-BSA 1%, cells were fixed in PBS-paraformaldehyde 4% for 1 h on ice. Apoptotic cells were detected with the Apoptosis Detection System (Promega, Madison, WI, USA). Briefly, cells were resuspended in 70% ice-cold ethanol solution and stored overnight at -20°C. Samples were labelled with TdT buffer according to the manufacturer's instructions. Reactions were terminated by adding EDTA. Cells were analyzed by flow cytometry (Elite Epics II, Coulter, Hialeah, FL, USA) for the amount of fluorescein-12-dUTP. In order to identify both apoptotic and non-apoptotic events, cells were resuspended in propidium iodide solution (diluted to 5 μ g/ml in PBS) and analyzed by flow cytometry.

3. Results

3.1. Effects of palmitoylated PKC pseudosubstrate lipopeptides on the phosphorylation of their isoenzymes

PKC- α , - ϵ and - ζ pseudosubstrate lipopeptides were assessed for their ability to inhibit the activity of the purified recombinant PKC- α , - ϵ or - ζ . Only inhibition of PKC- ζ was selective for its pseudosubstrate (Fig. 1). A clear dose-dependent inhibitory effect was observed with the PKC- ζ pseudosubstrate lipopeptide (IC₅₀: 0.3 μ M), while neither the scrambled lipopeptide nor the PKC- α or PKC- ϵ pseudosubstrate lipopeptides exhibited inhibitory activity upon purified recombinant PKC- ζ . Inhibition of the purified recombinant PKC- α or PKC- ϵ was observed with all three palmitoylated pseudosubstrate lipopeptides, revealing no selectivity for the PKC isoenzymes (data not shown). Since cell-free systems do not contain crucial components relevant to the phenomenon under study, we elected to review the selectivity of each palmitoylated pseudosubstrate lipopeptide in assays to be performed on intact cells.

3.2. Expression and subcellular localization of PKC isoenzymes

The expression and the subcellular localization of PKC- α , - ϵ and - ζ in Jurkat cell line was determined by confocal microscopy (Fig. 2) using monoclonal antibodies directed against PKC- α , - ϵ or - ζ and showed a cytoplasmic expression for all three isoenzymes. However, the subcellular localization of each isoenzyme was different. Antibodies directed against PKC- α revealed high intensity cytoplasmic staining in close proximity to the plasma membrane. This staining was not diffuse but localized in clusters (Fig. 2A). In contrast, the immunostaining of PKC- ζ showed a uniform and diffuse cytoplasmic labelling (Fig. 2E). The identical feature of cellular distribution was observed for PKC- ϵ (data not shown). No nuclear expression was detected for the three PKC isoenzymes.

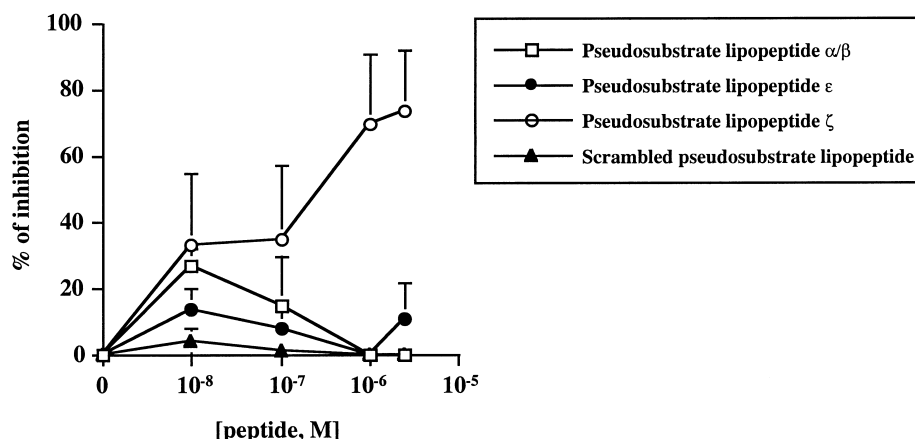


Fig. 1. Inhibition of kinase activity of purified recombinant PKC- ζ incubated with either PKC- α/β (\square), - ϵ (\bullet) or - ζ (\circ) pseudosubstrate lipopeptides or scrambled pseudosubstrate lipopeptide (\blacktriangle). Mean of percentage of inhibition \pm S.E.M. ($n=4$).

3.3. Intracellular delivery of PKC pseudosubstrate lipopeptides and colocalization with their respective PKC isoenzymes

To address the intracellular delivery of the pseudosubstrate lipopeptides and their respective cellular localization, we used the pseudosubstrate lipopeptides of each isoenzyme, modified at their N-terminus by a biotin group.

The intracellular localization of the biotinylated lipopeptides was proven since the cells needed to be permeabilized before Alexa 488-streptavidin treatment in order to achieve fluorescent labelling. No fluorescence was observed in cells that were not permeabilized (data not shown). This observation dismisses the possibility that the lipopeptide was anchored to the extracellular part of the cell membrane. The internalization of lipopeptides occurs rapidly, since following a 10 min incubation, lipopeptides were observed in the cells (Fig. 2B,F). Furthermore, this cytoplasmic delivery does not require endocytosis, as a 4°C incubation does not affect the observed cytoplasmic delivery (data not shown).

When cells were incubated with the biotinylated PKC- α , - ϵ or - ζ pseudosubstrate lipopeptides, and then stained with monoclonal antibodies directed against the same PKC isoforms, an overlapping signal was observed. Indeed, Jurkat cells incubated with PKC- α biotinylated pseudosubstrate lipopeptide, then stained with a monoclonal antibody directed against PKC- α , exhibited an overlapping signal characterized by intracellular clusters near the plasma membrane, attesting to a colocalization of the lipopeptide and its defined target (Fig. 2D). In addition, when Jurkat cells were incubated with PKC- ζ biotinylated pseudosubstrate lipopeptide, then stained with a monoclonal antibody directed against PKC- ζ , the overlapping signal was diffuse in the cytoplasm, following the subcellular localization of the PKC isoenzyme (Fig. 2H). A similar pattern of colocalization was observed with cells incubated with PKC- ϵ biotinylated pseudosubstrate lipopeptide, and then stained with a monoclonal antibody directed against PKC- ϵ (data not shown).

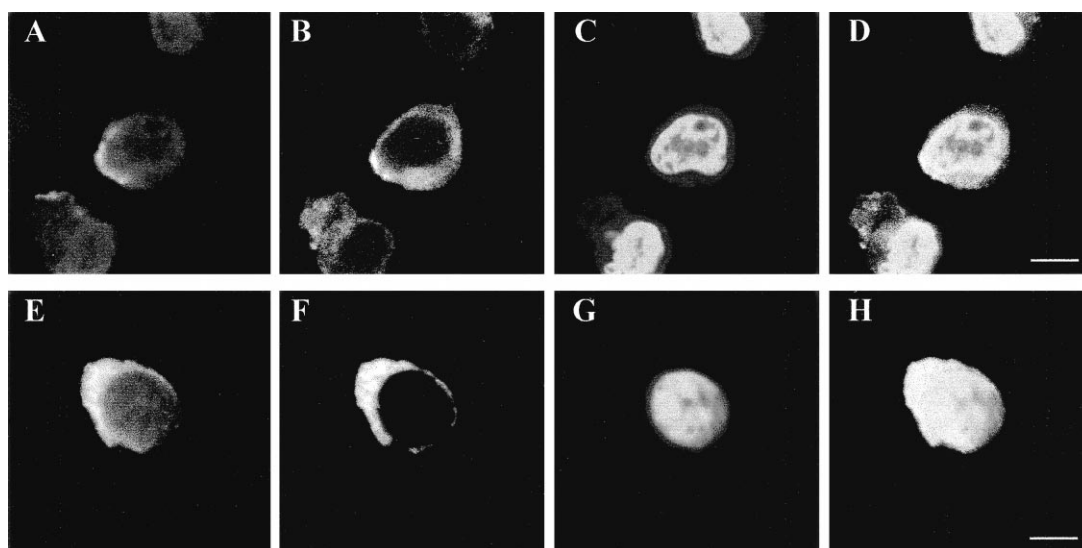


Fig. 2. Confocal microscopy study of the cellular distribution of PKC- α and - ζ isoenzymes together with their respective pseudosubstrate lipopeptides. PKC isoenzymes were detected by indirect immunofluorescence using a monoclonal antibody against PKC- α or - ζ and a goat anti-mouse Alexa 568 conjugated as secondary antibody (A and E). Cellular localization of biotinylated lipopeptides was revealed with Alexa 488-streptavidin (B and F). Nuclear DNA was stained with TO-PRO-3 iodide (C and G). Triple exposure (D and H) show the overlapping fluorescence of the different pseudosubstrate lipopeptides and their respective PKC isoenzymes. Magnification: $\times 1000$. Bar equals 5 μ m.

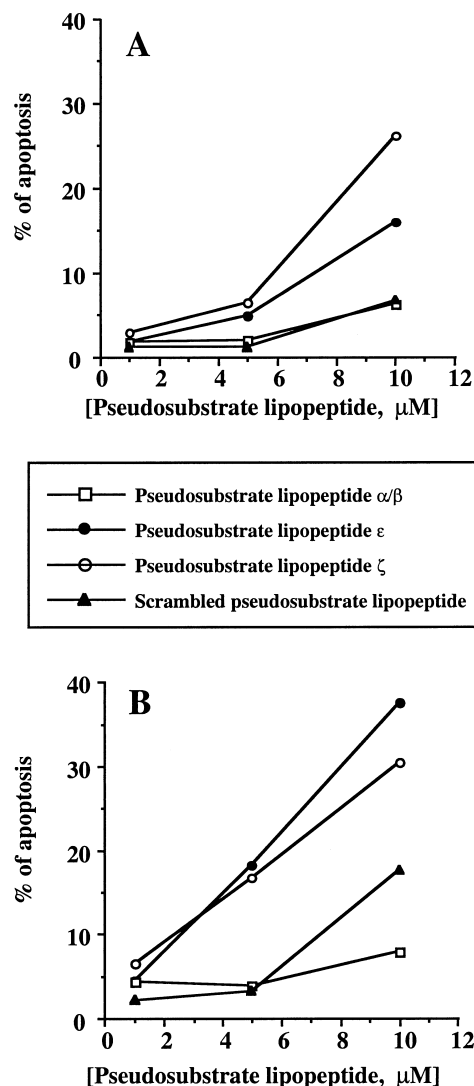


Fig. 3. Quantification of apoptosis induced on Jurkat cells (A) and HL-60 cells (B) incubated for 6 h with either PKC- α/β (\square), - ϵ (\bullet) or - ζ (\circ) pseudosubstrate lipopeptides or scrambled pseudosubstrate lipopeptide (\blacktriangle). Apoptosis was quantified by flow cytometry analysis using the TUNEL methodology. Results are representative of three independent experiments.

3.4. Effect of different PKC isoenzyme pseudosubstrate lipopeptides on apoptosis

We next wanted to assess the biological functionality of the pseudosubstrate lipopeptides. In a previous report [20] we described that treatment of several human cell lines with 50 μM of PKC- α/β pseudosubstrate lipopeptide resulted in the induction of apoptosis. Since a key role is indicated for PKC in the mechanism of apoptosis, we decided to investigate the potential effect of PKC- α , - ϵ or - ζ pseudosubstrate lipopeptides upon the induction of apoptosis in human cells. Apoptosis in treated cells was qualitatively observed by DNA fragmentation (data not shown) and quantified by flow cytometry (Fig. 3). Jurkat cells incubated with different concentrations of either PKC- ϵ or - ζ pseudosubstrate lipopeptides for 6 h, exhibited an increase of apoptosis in a dose-dependent manner (Fig. 3A). This induction of apoptosis was not related to palmitic acid since the control scrambled lipopeptide did not

exhibit such an effect. Furthermore, this induction of apoptosis was dependent upon the pseudosubstrate sequence as PKC- α/β pseudosubstrate lipopeptide or scrambled lipopeptide did not lead to a significant induction of apoptosis. A similar study performed on the HL60 cell line (Fig. 3B) revealed a greater sensitivity of these cells to apoptosis, since apoptosis was induced by lower concentrations (5 μM) of PKC- ϵ or - ζ pseudosubstrate, whereas neither the PKC- α/β pseudosubstrate lipopeptide nor the scrambled lipopeptide showed comparable effects. In this cellular assay the induction of apoptosis induced by either PKC- ϵ or - ζ pseudosubstrate was remarkable starting at 5 μM , and increasing in a dose-dependent manner.

4. Discussion

In a previous report [19] we showed that palmitic modification of PKC- α/β pseudosubstrate increased the ability of the pseudosubstrate to inhibit the phosphorylation of the corresponding PKC isoenzymes. Moreover, we reported [19,20] that incubation of intact cells with PKC- α/β pseudosubstrate lipopeptide led to the inhibition of biological activities known to be mediated by PKCs, whereas the non-lipidic PKC- α/β pseudosubstrate did not exhibit similar effects suggesting an intracellular delivery of the lipopeptide. Thus, we decided to modify PKC- α/β , - ϵ , - ζ pseudosubstrate sequences with a palmitic moiety in order to allow their cytoplasmic delivery and to investigate their implication in the induction of apoptosis.

In an attempt to evaluate the inhibitory activity of each pseudosubstrate lipopeptide with its respective purified isoenzyme, we studied their ability to inhibit kinase activity in a cell-free enzyme assay. Only PKC- ζ was selectively inhibited, in a dose-dependent manner, by its pseudosubstrate lipopeptide. Since Zoukhri et al. [23] reported that no selectivity of PKC pseudosubstrate sequences was observed in a cell-free system, while results obtained in cellular assay argue in favor of the selective inhibition of PKC isoenzymes, we decided to investigate the efficiency of the different pseudosubstrate lipopeptides to modulate the activity of their corresponding isoenzymes in a cellular assay.

First, we clearly established the intracellular delivery of the palmitoyl modified pseudosubstrate of three different PKC isoenzymes: PKC- α , - ϵ and - ζ . We determined by confocal microscopy the subcellular localization of the three PKCs isoenzymes in the Jurkat cell line. Then, we investigated the cellular distribution of different PKC pseudosubstrate lipopeptides. We found that PKC- α pseudosubstrate lipopeptide was located intracellularly near the cell membrane and double staining revealed its colocalization with PKC- α . The same experiments performed with PKC- ζ and - ϵ pseudosubstrate lipopeptides revealed a diffuse cytosolic distribution of the two lipopeptides which corresponded to their respective PKC isoenzymes. These results confirmed the different subcellular localization of PKC isoenzymes as previously reported [24]. When we studied the distribution of PKC- ζ with PKC- α pseudosubstrate lipopeptide, the latter was always observed in proximity to the inner surface of the cell membrane, where its defined target is localized. Nevertheless, since localization of the PKC- ζ is diffuse into the cytoplasm, double exposure observation reveals a weak overlapping signal adjacent to the cell membrane (data not shown). The same feature of observation was obtained when we examined the distribution of

PKC- ϵ with PKC- α pseudosubstrate lipopeptide, since PKC- ϵ is also diffused into the cytoplasm [24].

Since PKCs play a key role in apoptosis, the modulation of different isoenzymes was evaluated through the quantification of apoptosis in cells treated with PKC- α , - ϵ and - ζ pseudosubstrate lipopeptides. Induction of apoptosis was clearly dependent upon the pseudosubstrate sequence, attesting to a selective effect on apoptosis for each PKC isoenzyme. Indeed, the human T cell line (Jurkat) showed a greater sensitivity to apoptosis induced by PKC- ζ pseudosubstrate and a reduced sensitivity to PKC- ϵ pseudosubstrate lipopeptide, whereas the same concentrations (5 μ M, 10 μ M) of PKC- α/β pseudosubstrate lipopeptide did not induce programmed cell death. The human leukemia cell line (HL-60) was highly sensitive to apoptosis induced by PKC- ϵ pseudosubstrate lipopeptide and to a lesser extent the PKC- ζ pseudosubstrate lipopeptide. Neither PKC- α/β pseudosubstrate lipopeptide nor scrambled control lipopeptide exhibited comparable biological activities. Taken together these results suggest a selective activity of PKC pseudosubstrates which might be related to a similar selective action on their respective isoenzyme. These data dismiss the results obtained in a cell-free assay where PKC pseudosubstrate lipopeptides were evaluated for their ability to selectively inhibit the activity of their respective isoenzyme. Indeed, the results obtained established that purified recombinant PKC- ζ was the only isoenzyme found to be selectively inhibited by its pseudosubstrate. Based upon the cell-free assay, one would expect that in intact cells, a co-localization PKC- α/β pseudosubstrate with either PKC- α and - ϵ isoenzymes would be observed. However, the colocalization of the lipopeptide was clearly associated with the distribution of its target (PKC- α). Moreover, when human cells were incubated with 5 μ M of palmitoylated PKC- ζ pseudosubstrate plus 5 μ M of palmitoylated PKC- ϵ pseudosubstrate, the apoptosis observed was not comparable to that induced by 10 μ M of either PKC- ζ or - ϵ pseudosubstrate, but was similar to that induced by 5 μ M of each lipopeptide (data not shown). The discrepancy in the selectivity observed between the cellular assay and the cell-free assay might be related to the absence of some crucial components for the phenomenon under study. Indeed, increasing evidence suggests that part of the specificity of action of PKC isoenzymes might be conferred by their association with specific proteins that target them to specific intracellular compartments [8,25–28]. Moreover, our results concerning the induction of apoptosis by PKC- ϵ and PKC- ζ pseudosubstrate lipopeptides confirmed previous reports where these two isoenzymes were involved in cell survival mechanisms. Pongracz et al. [29] indicated that the expression of specific PKC isoenzymes is modulated during apoptosis and that PKC- β and PKC- ζ may play specific roles in the regulation of the apoptotic program. The latter isoenzyme has been identified to be closely involved in anti-apoptotic processes [30–33]. PKC- ϵ is also reported to be a reducer of apoptosis [34] and a putative oncogene [35,36]. Recently Mayne et al. [37] showed that PKC- ϵ is required for the protective effect of TPA in tumor necrosis factor α -induced apoptosis suggesting that PKC- ϵ may play an important role in leukemia cell survival. On the other hand, Gubina et al. [38] reported that overexpression of PKC- ϵ in an interleukin-3-dependent human cell prolonged cell survival in the absence of the cytokine and resulted in an induction of bcl-2 expression. All these data converge with our observations concern-

ing the induction of apoptosis with PKC- ϵ and PKC- ζ pseudosubstrate lipopeptide-treated cells. Taken together with the colocalization of these pseudosubstrates and their specific targets, our work strengthens the hypothesis that the two isoenzymes are involved in cell survival mechanisms. Implication of PKC- α in an anti-apoptotic mechanism seems to be of less importance, since at the concentrations used its pseudosubstrate lipopeptide did not induce apoptosis but higher concentrations of PKC- α pseudosubstrate lipopeptide led to a specific induction of apoptosis as previously described [20]. These data argue in favor of a differential implication of the three PKC isoenzymes in the control of apoptosis.

Besides the modulation of PKC isoenzymes, our work emphasizes the use of lipopeptides to modulate cellular activities through an interaction with intracytoplasmic targets. Indeed, the ability of lipopeptides to be intracellularly delivered while remaining biologically active gives rise to new opportunities. In fact, in other reports [39,40] we described the synthesis of a lipopeptide agonist toward interferon- γ receptor. These synthetic agonists of a cytokine were able to reproduce the activities of the native cytokine, via interaction with the intracellular part of the cytokine receptor. Taken together, these different reports [19,20,39,40] emphasize the use of lipopeptides as a promising new approach for the pharmacomodulation of intracellular targets.

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